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Development of Pyrethroid Substrates for Esterases Associated with Pyrethroid Resistance in the Tobacco Budworm, *Heliothis virescens* (F.)

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Assays to detect esterases associated with resistance to organophosphorus and pyrethroid insecticides in larvae of *H. virescens* were developed and evaluated. Cross-resistance to a variety of insecticides was measured in strains resulting from selection with either profenofos (OP-R) or cypermethrin (PYR-R), and resistance in both strains appeared to have a metabolic component. Esters were synthesized that coupled 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, the acid moiety of some pyrethroid insecticides, with groups (e.g., *p*-nitrophenyl-) that could be detected spectrophotometrically following hydrolysis of the resulting esters. Activities toward these pyrethroid esters were significantly higher in both resistant strains than those in a susceptible reference strain. In addition, all pyrethroid esters significantly increased the toxicity of cypermethrin in bioassays with larvae from both PYR-R and OP-R strains. The biological and biochemical activities of these compounds are compared with those with more conventional esterase substrates and insecticide synergists, and the utility of pyrethroid esters as components of rapid assays for detecting esterases associated with insecticide resistance is discussed.

KEYWORDS: Tobacco budworm; pyrethroid esters; insecticide synergists; cross-resistance; esterases

INTRODUCTION

Insecticide resistance is a major impediment to the effective management of field populations of the tobacco budworm, *Heliothis virescens* (*1*−*3*). Resistance may result from enhanced enzymatic detoxication or reduced penetration of the insecticide and/or reduced sensitivity at the site of action of an insecticide (*4*, *5*). Expression of all three resistance mechanisms has been reported in *H. virescens* (*2*, *3*, *6*).

The ability to discriminate among these three biochemical mechanisms is essential for the development and optimization of insecticide resistance management strategies. At present, there are a number of techniques available to identify insecticide resistance mechanisms (*7*); however, most methodologies are designed for use in the laboratory and are difficult to utilize in field situations without modification. Insecticide bioassays with synergists are fast, easy to use, and have a potential field utility, but the specificity of these techniques is often questionable because multiple forms of detoxifying enzymes may be expressed concurrently in resistant insects (*8*), and the specificity of these enzymes toward a single inhibitor is variable (*9*, *10*). Therefore, the lack of synergism of insecticide toxicity might indicate that either a detoxifying enzyme is not responsible for resistance or a resistance-associated enzyme is present but not inhibited by the synergist used (*11*).

In addition to synergist bioassays, colorimetric measurements of enzyme activities have been used to detect changes in detoxifying enzymes expressed in insecticide resistant insects. The disadvantages of these assays are similar to those of assays with insecticide synergists: Model (i.e., noninsecticide) substrates may not be selectively metabolized by enzymes responsible for resistance. For example, 1-naphthyl acetate (1-NA), which has been used extensively as a model substrate in resistance studies, is a poor indicator of resistance-associated esterases from pyrethroid resistant *H. virescens* (*12*).

Developing more specific synergists and substrates for resistance-associated enzymes would enhance the accuracy of assays for detecting resistance. Our working hypothesis is that the specificity with which resistance-associated esterases are detected may be enhanced by using reagents that are structurally similar to the insecticide that is resisted. The approach used was to modify the structure of a commonly used pyrethroid insecticide such that the product of hydrolysis is detectable by spectrophotometry. A similar approach has been used to study esterases associated with pyrethroid hydrolysis in the cattle tick *Boophilus microplus* (*13*) and in human serum (*14*). The major objective of this study was to synthesize pyrethroid esters and test them both as synergists of pyrethroid toxicity and as substrates for esterases in insecticide susceptible and resistant strains of *H. virescens*.

MATERIALS AND METHODS

Chemicals. Permethrin (94.6%), bifenthrin (96%), tefluthrin (97%), cypermethrin (96%), and acephate (97.6%) were kindly provided by FMC Corporation (Princeton, NJ). Technical grade profenofos (89%)

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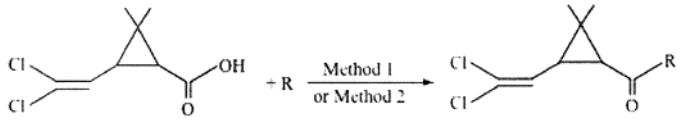
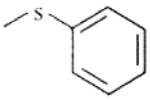
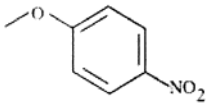
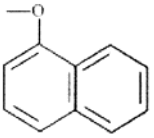
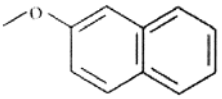
Table 1. Structures and Physical Characteristics of Pyrethroid Esters



designation	R	purity (%)[a]	Rt (min)[b]	m/z (RI)[c]
TPPA		98.6	18.19 (*cis*) 18.36 (*trans*)	*cis*: 127.0 (100), 191.1 (88.7), 91.1 (84.9), 63.0 (65.9), 193.1 (65.3), 165.0 (40.0), 129.0 (38.6), 241.0 (10.9), 77.0 (13.3). *trans*: 127.0 (100), 163.0 (74.1), 91.1 (74.0), 191.1 (71.9), 165.1 (48.4).
p-NPPA		96.4	10.07 (*cis*) 10.19 (*trans*)	*cis*: 187.1 (100), 127.1 (53.8) 91.1 (49.8), 225.0 (39.9), 227.0 (35.1), 189.1 (33.4). *trans*: 187.1 (100), 127.1 (58.8), 91.1 (40.5), 189.0 (34.7), 163.0 (25.9).
1-NPA		98.7	21.34 (*cis*) 21.52 (*trans*)	*cis*: 191.0 (100), 127.0 (88.2), 193.0 (68.6), 91.1 (53.0), 115.1 (41.7), 129.0 (29.4), 225.0 (26.2). *trans*: 191.0 (100), 127.1 (89.1), 193.0 (69.3), 91.1 (58.7), 225.0 (40.0), 115.1 (31.1), 129.0 (29.5).
2-NPA		92.1	21.85 (*cis*) 22.03 (*trans*)	*cis*: 127.1 (100), 191.0 (87.3), 91.1 (68.7), 225.0 (59.7), 193.0 (58.7), 144.1 (57.3), 209.0 (47.1), 129.1 (31.6). *trans*: 127.1 (100), 191.0 (89.41), 225.0 (87.5), 193.0 (77.1), 91.1 (65.4), 209.0 (65.3), 144.1 (60.6), 129.0 (31.4).

[a] Purity was calculated based on relative peak area. [b] R_t = retention time. [c] RI = relative intensity, percent.

was a gift from Novartis (Greensboro, NC). Indoxacarb (100%) and spinosyn A (100%) were gifts from DuPont Agricultural Products (Newark, DE) and DowElanco Corporation (Indianapolis, IN), respectively. *trans*-Fenfluthrin and 2,3,6-trichloro-3-(2-propynyloxy)benzene (TCPB; >95% purity) were synthesized as described previously (*15*) and recrystallized before use. *S*,*S*,*S*-Tributylphosphorotrithioate (DEF; 98.7% purity) was provided by Bayer Corporation (Kansas City, MO), and piperonyl butoxide (PBO; 90−95% purity) was purchased from Fluka Chemical Corporation (Milwaukee, WI). Fast Blue B salt (90% purity), 1-NA (98% purity), benzenethiol (97% purity), *S*-methyl thiobutanoate (SMTB, 98% purity), 1-naphthol (99% purity), 2-naphthol (99% purity), acetyl chloride (97% purity), thionyl chloride (99% purity), phosphorus pentoxide (98% purity), 5,5′-dithio bis(2-nitrobenzoic acid) (DTNB, 99% purity), 4-nitrophenol (99% purity), and benzoyl chloride (99% purity) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Silica gel (28−200 mesh, average pore diameter 22 Å) and 2-naphthyl acetate were purchased from Sigma Chemical Co., Inc. (St. Louis, MO). The methyl ester of 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (PA; cis/trans = 40/60) was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were of analytical quality and were purchased from commercial suppliers.

Synthesis of Pyrethroid Esters. Pyrethroid esters that were synthesized and tested include the following: thiophenyl, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (TPPA), *p*-nitrophenyl, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (*p*-NPPA), and 1- or 2-naphthyl, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (1- or 2-NPA; **Table 1**). The preparation of PA from its methyl ester followed the procedure of Shan et al. (*15*). A mixture of the methyl ester of PA (22.3 g, 0.1 mol), sodium hydroxide (12 g, 0.3 mol), and ethanol−water (1:1, 250 mL) was refluxed for 12 h, concentrated under reduced pressure, diluted with 150 mL of water, and extracted with ethyl ether (3 × 30 mL). The aqueous phase was acidified with HCl (37.4%, 30 mL), and the oily precipitate was extracted into ether (3 × 30 mL), washed with water (2 × 25 mL), and then dried over anhydrous Na_2SO_4 overnight. The solution was filtered, and ethyl ether was evaporated under reduced pressure to obtain a solid product, which was recrystallized from hexane/ether (1:1, v/v); yield, 18.0 g (86%).

The trans and cis isomers of PA were separated using the method of Foggassy et al. (*16*) with modifications. A mixture of PA (20.9 g, 0.1 mol) and benzene (100 mL) was stirred at 27 °C for 5 h. The suspension was filtered, and the filter residue was recrystallized from benzene to give 5.0 g of pure *cis*-PA (60% recovery). After benzene was removed from the filtrate, the solid product was stirred for 5 h at 27 °C in 100 mL of petroleum ether and then filtered to give 2.5 g of a solid product, which was recrystallized from hexane to give 1.8 g of pure *trans*-PA (14% recovery).

Two methods were used to prepare esters from PA. First, thionyl chloride (0.1 mol, 8.7 mL) was added dropwise into a mixture of PA (0.025 mol, 5.25 g) and 100 mL of benzene (60% in hexane) and then refluxed for 3 h at 50−60 °C. The solvent and excess thionyl chloride were removed from the reaction mixture, and an oily product (PA-chloride) was obtained (*13*). Freshly prepared PA-Cl (0.025 mol) was added to a mixture containing 50 mL of benzene (60% in hexane) and *p*-nitrophenol (3.5 g, 0.025 mol) and refluxed for 4 h at 50−60 °C. The reaction mixture was washed with saturated $NaHCO_3$ (20 mL × 3) and 50% ethanol−water (50 mL × 2), and then purified by column chromatography (silica gel; 28−200 mesh, average pore diameter 22 Å) with hexane:ethyl acetate (70:30) as the developing phase to obtain 5.2 g of *p*-NPPA (62.5% yield). A similar method was used to prepare thiophenyl acetate (TPA) and the 1- or 2-naphthyl esters of PA (1-NPA or 2-NPA). After they were washed with $NaHCO_3$ (20 mL × 3) and 50% ethanol−water (50 mL × 2), the final reaction mixtures were purified by column chromatography using hexane:ethyl acetate (80:20 for TPA or 100:10 for NPA esters). The yields of TPA, 1-NPA, and 2-NPA were 72, 79.7, and 80.5%, respectively.

A second method (direct esterification between an acid and an alcohol under dehydrating conditions) was used to prepare TPPA (*17*, *18*). A reaction mixture containing *cis*- or *trans*-PA (0.01 mol), thiophenol (0.011 mol), polyphosphate ester (20 mL), chloroform (80 mL), and pyridine (10 drops) was stirred for 12 h at room temperature. The final mixture was washed with saturated $NaHCO_3$ (2 × 50 mL)

and then extracted with chloroform (2 × 40 mL). The combined organic phase was dried over anhydrous Na_2SO_4 overnight. After the solvent was removed, the solid product was purified by column chromatography (silica gel; 28−200 mesh, average pore diameter 22 Å) with a mixture of hexane:ethyl acetate (70:30) as the developing phase. The yields of *cis*-and *trans*-TPPA were 7.30 (96.5%) and 6.95 g (93.0%), respectively.

Identification of Products. The products were identified using gas chromatography−mass spectrometry (GC-MS) on a Finnigan GCQ (Trace GC 2000 coupled with Polaris mass spectral detector) (**Table 1**). A silica capillary GC column (DB-5MS; 30 m × 0.25 mm × 0.25 μm; J &W Scientific, Folsom, CA) was operated at 60 °C for 1 min, increased to 150 °C at 5 °C/min increments, held for 15 min at this temperature, and then increased to 300 °C at a rate of 5 °C/min, where it was held for the final 10 min. The injector port was operated in the splitless mode at 200 °C, and helium was used as the carrier gas at 1.2 mL/min. The mass spectral detector was set on full scan mode (*m*/*z* 41−400). The purity was calculated by relative peak area.

Insects. A susceptible strain of *H. virescens* (LSU-S) was established in 1977 (*19*) and has been reared in the laboratory since that time without exposure to insecticides. Larvae from the LSU-S strain were selected for five consecutive generations with topical applications of profenofos at doses corresponding to the LD_{60} for each generation (3.5, 4.6, 8.5, 13.6, and 23.8 μg/individual). After 1 year without selection, individual larvae were treated with 25 μg of profenofos (an approximate LD_{20}) and the survivors (OP-R strain) were used for biological assays. Similarly, larvae from the LSU-S strain were selected for four consecutive generations with topical applications of cypermethrin at doses corresponding to the LD_{60} (0.22, 0.4, 0.5, and 0.6 μg/individual). After one generation without selection, larvae were treated with 1 μg of cypermethrin (an approximate LD_{90}), and the next generation (PYR-R strain) was used for biochemical and biological assays.

Toxicological Assays. Fifth instars (day 1), weighing 180 ± 20 mg, were treated on the midthoracic dorsum with 1 μL of insecticide (in acetone) or with acetone alone (control). Dose−mortality relationships for each insecticide were assessed from assays with at least five doses and 30 insects per dose. To evaluate the effects of conventional synergists (i.e., PBO, DEF, and TCPB) and pyrethroid esters on the toxicity of cypermethrin or profenofos, compounds were applied to the midabdominal dorsum 30 min prior to application of the insecticide. In preliminary assays, doses (0−60 μg/larva) of the five pyrethroid esters (1- or 2-NPA, *cis*- or *trans*-TPPA, and *p*-NPPA) were topically applied to larvae from the LSU-S strain, followed by a dose of cypermethrin corresponding to the LD_{20} (0.15 μg/larva). Doses of the pyrethroid esters causing maximum mortality in these tests were used in assays with resistant strains. All pyrethroid esters were nontoxic to LSU-S larvae at 70 μg/larva. In tests with PBO, DEF, and TCPB, a dose of 50 μg/larva was used (*15*). For all bioassays, larvae that were treated with either 1 μL of acetone or the synergist alone served as controls. After treatment, larvae were maintained at 27 °C, and mortality was recorded after 72 h. The criterion for mortality was the lack of coordinated movement within 30 s after being prodded with a sharpened pencil. The results were corrected for control mortality with Abbott's formula (*20*) and then analyzed by Finney's method (*21*). The differences between LD_{50} values were considered significant if the 95% fiducial limits (FLs) did not overlap. Resistance ratios (RR) were calculated as LD_{50} of resistant strain/LD_{50} of susceptible strain. The synergism ratio (SR) was calculated as: LD_{50} of insecticide alone/LD_{50} of insecticide plus synergist.

Biochemical Assays. Homogenates from individual fifth instars were used as the enzyme source for measuring esterase activities toward all substrates. Individual larvae were dissected, and the digestive system was evacuated. The remaining carcass was homogenized using an all-glass homogenizer with 500 μL of ice-cold, 0.1 M sodium phosphate buffer (pH 6−8). Homogenates were centrifuged at 12600*g* for 15 min at 4 °C, and the resulting supernatants were held in ice and used in enzyme assays within 20 min of preparation. For all substrates, assay conditions (i.e., pH, temperature, substrate concentration, and protein dependence) were optimized in preliminary experiments (data not shown). Activities were measured in 96 well microplates using a Thermomax Plate Reader (Molecular Devices, Palo Alto, CA) and were corrected for nonenzymatic hydrolysis using reactions without protein as the control.

Activities of esterases toward 1-NA, 1-NPA, and 2-NPA were measured by modifying the method of Gomori (*22*). Reaction mixtures contained (final concentration in 250 μL): homogenate (60 μg of protein), phosphate buffer (0.1 mM; pH 7.0), and a solution containing Fast Blue B (1.2 mM) and substrate (2.15, 0.67, or 0.67 mM for 1-NA, 1-NPA, or 2-NPA, respectively). Changes in optical density at 450 nm were measured during the initial 10 min of the reactions, and rates were converted to nmol min^{-1} using the extinction coefficient 9.25 mM^{-1} 250 μL^{-1} for 1-naphthol (*23*). Esterase activities toward SMTB, TPA, and *cis*- or *trans*-TPPA were measured by the method of Ellman et al. (*24*), as modified by van Asperen (*25*). Reaction mixtures contained (final concentrations in 300 μL): homogenate (30, 30, or 120 μg of protein for SMTB, TPA, or *cis*-/*trans*-TPPA, respectively), DTNB (0.05 mM), phosphate buffer (0.1 mM; pH 7.6, 7.6 or 8.0 for SMTB, *cis*-/*trans*-TPPA, or TPA, respectively), and substrate (2.0, 1.33, or 1.33 for SMTB, *cis*-/*trans*-TPPA, or TPA, respectively). Changes in optical density at 405 nm were measured during the initial 10 min (or 1 min for TPA) of the reactions, and rates were converted to nmol min^{-1} using an experimentally derived "extinction coefficient" of 8.34 mM^{-1} 300 μL^{-1}. Finally, the esterase activity toward *p*-NPPA was measured using the method of Kinoshita et al. (*26*) as modified by Hansen and Hodgson (*27*). The reaction mixtures contained (final concentrations in 300 μL): homogenate (360 μg of protein), phosphate buffer (0.1 mM; pH 7.6), and substrate (1.33 mM). Changes in optical density at 405 nm were measured during the first 10 min of the reactions, and rates were converted to nmol min^{-1} using an experimentally derived extinction coefficient of 11.4 mM^{-1} 300 μL^{-1} for *p*-nitrophenol. Homogenates from at least 30 larvae were used in assays with each substrate. Protein concentrations were measured from diluted homogenates by the method of Bradford (*28*) using bovine serum albumin as the standard. Results were analyzed and compared using analysis of variance (ANOVA)−Tukey's HSD test ($P \leq 0.01$).

RESULTS

Characterization of Resistant Strains. The selection of LSU-S larvae with either profenofos (OP-R strain) or cypermethrin (PYR-R) resulted in resistance to all insecticides tested except acephate in the PYR-R strain (**Table 2**). Resistance was highest to the insecticide used as the selecting agent (i.e., 18.1-fold resistance to profenofos in the OP-R strain and 19.6-fold resistance to cypermethrin in the PYR-R strain). In tests with OP-R larvae, resistance was also high to indoxacarb (15.8-fold) and to pyrethroids containing 3-phenoxybenzyl- groups (i.e., 12.9-fold to cypermethrin and 7.2-fold to permethrin). Similarly, in cypermethrin-selected (PYR-R) larvae, resistance was also high to permethrin (12.4-fold) but lower for pyrethroids with nonphenoxybenzyl alcohols such as bifenthrin (3-phenyl 2-methylbenzyl alcohol; 5.1-fold), fenfluthrin (pentafluorobenzyl alcohol; 2.91-fold), or tefluthrin (4-methyl tetrafluorobenzyl alcohol; 2.18-fold). Finally, as compared with OP-R larvae, cross-resistance to profenofos and indoxacarb was lower (4.53- and 5.57-fold, respectively) in larvae from the PYR-R strain.

Synergism of Insecticide Toxicity. All of the pyrethroid esters tested synergized the toxicity of cypermethrin to LSU larvae (**Figure 1**). The NPA esters were slightly more potent than the TPPA compounds, and differences in potencies of the geometric (for TPPA) and positional isomers (for NPA) were minimal. Maximal levels of mortality (50−63%) were similar among compounds within the range of doses tested, but optimal doses for synergism differed and ranged from 20 (for 1- and 2-NPA) to 50 μg/larva (for *p*-NPPA).

Effects of nonpyrethroid compounds (i.e., DEF, PBO, and TCPB) on insecticide toxicity varied depending on the insect strain and insecticide used for assays (**Table 3**). In tests with the LSU-S strain, PBO synergized cypermethrin toxicity but was an antagonist of profenofos toxicity. In contrast, PBO had

Table 2. Insecticide Susceptibility of Larval *H. virescens* from Insecticide Susceptible (LSU-S) and Resistant (OP-R and PYR-R) Strains

strain[a]	insecticide	LD_{50} (95% FL[b])	slope (SD[c])	χ-square	RR[d]
LSU-S	profenofos	2.90 (2.40–3.56)	2.63 (0.40)	1.38	1
	cypermethrin	0.19 (0.16–0.24)	2.81 (0.39)	2.90	1
	permethrin	0.14 (0.11–0.19)	1.91 (0.25)	0.93	1
	tefluthrin	0.51 (0.42–0.69)	2.65 (0.52)	0.05	1
	trans-fenfluthrin	1.64 (1.22–2.12)	2.99 (0.40)	3.73	1
	bifenthrin	0.05 (0.04–0.06)	3.12 (0.42)	3.08	1
	indoxacarb	0.27 (0.22–0.33)	4.46 (0.64)	2.05	1
	acephate	25.8 (22.3–29.4)	4.26 (0.61)	1.65	1
	spinosyn A	1.84 (1.35–2.66)	1.66 (0.29)	0.49	1
OP-R	profenofos	52.3 (46.0–59.0)[e]	3.88 (0.57)	1.76	18.1
	cypermethrin	2.46 (1.86–3.43)[e]	6.49 (0.88)	1.06	12.9
	permethrin	1.03 (0.54–1.75)[e]	2.05 (0.31)	3.99	7.20
	tefluthrin	1.68 (1.38–2.01)[e]	3.00 (0.42)	2.38	3.30
	trans-fenfluthrin	4.30 (3.70–4.88)[e]	4.94 (0.64)	0.16	2.59
	bifenthrin	0.17 (0.13–0.24)[e]	1.77 (0.34)	2.03	3.47
	indoxacarb	4.29 (3.18–5.87)[e]	1.62 (0.23)	1.11	15.8
	acephate	69.1 (55.0–86.8)[e]	3.64 (0.53)	4.76	2.76
	spinosyn A	4.03 (2.85–5.49)[e]	1.52 (0.22)	0.64	2.19
PYR-R	profenofos	13.2 (10.4–16.2)[e]	2.49 (0.39)	2.17	4.53
	cypermethrin	3.75 (3.16–4.33)[e]	3.34 (0.48)	1.56	19.6
	permethrin	5.59 (4.27–7.91)[e]	1.77 (0.28)	0.57	12.4
	tefluthrin	1.11 (0.96–1.31)[e]	1.77 (0.28)	6.75	2.18
	trans-fenfluthrin	4.83 (3.91–6.25)[e]	4.49 (0.65)	3.18	2.91
	bifenthrin	0.26 (0.18–0.41)[e]	2.97 (0.38)	8.40	5.10
	indoxacarb	1.51 (1.02–2.06)[e]	1.68 (0.29)	0.44	5.57
	acephate	39.4 (24.8–62.5)	5.09 (0.61)	9.48	1.53
	spinosyn A	5.49 (3.79–7.93)[e]	1.30 (0.21)	0.19	2.99

[a] Strains: LSU-S, insecticide susceptible; OP-R, profenofos selected; PYR-R, cypermethrin selected. [b] FL = 95% fiducial limits. [c] SD = standard deviation. [d] RR is defined as LD_{50} of resistant strain/LD_{50} of LSU-S strain. [e] Denotes a value that is significantly different ($P \leq 0.05$) from the corresponding value for the LSU-S strain.

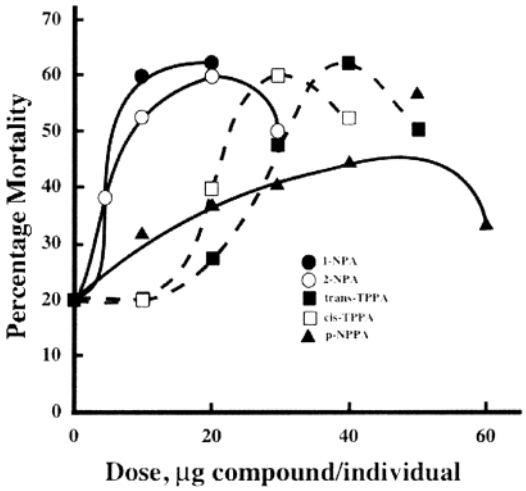


Figure 1. Mortality measured after sequential topical application of various doses of synergists followed by 0.15 μg of cypermethrin onto fifth stadium (day 1) larvae of *H. virescens* from the LSU-S strain.

no significant effect on the toxicity of cypermethrin or profenofos in either the OP-R or the PYR-R strains. Similarly, DEF antagonized profenofos toxicity in the LSU-S strain but had no significant effect on profenofos toxicity in the OP-R strain or on cypermethrin toxicity in the LSU-S or PYR-R strain. In contrast, TCPB was the most potent synergist of profenofos toxicity in the LSU-S and OP-R strains and synergized cypermethrin toxicity in PYR-R larvae but had no significant effect on cypermethrin toxicity in the LSU-S strain.

All pyrethroids esters significantly increased cypermethrin toxicity in both OP-R and PYR-R strains (**Table 3**). In the OP-R strain, SRs were similar among the five pyrethroid esters and ranged from 2.93 (for 1-NPA) to 3.67 (for *cis*-TPPA). Similarly, in tests with PYR-R larvae, SRs for the pyrethroid esters ranged from 1.80 (for 2-NPA) to 3.34 (for *p*-NPPA).

Table 3. Effects of Compounds on Profenofos or Cypermethrin Toxicity in Fifth Stadium Larvae from Susceptible (LSU-S) and Resistant (OP-R or PYR-R) Strains of *H. virescens*

strain[a]	treatment[b] (μg)	LD_{50} (95% FL)[c]	slope (SD)	SR[d]	RR[e]
LSU-S	cyper alone	0.19 (0.16–0.24)	2.81 (0.39)		
	cyper + PBO (50)	0.09 (0.08–0.10)[e]	4.02 (0.38)	2.05	
	cyper + DEF (50)	0.22 (0.16–0.38)	1.75 (0.34)	0.85	
	cyper + TCPB (50)	0.12 (0.08–0.18)	2.61 (0.36)	1.66	
	prof alone	2.90 (2.40–3.60)	2.63 (0.40)		
	prof + PBO (50)	7.24 (5.71–10.4)[e]	2.47 (0.53)	0.40	
	prof + DEF (50)	8.02 (6.52–11.2)[e]	2.06 (0.48)	0.36	
	prof + TCPB (50)	1.63 (1.23–1.97)[e]	2.93 (0.51)	1.78	
PYR-R	cyper alone	3.75 (3.16–4.33)	3.34 (0.48)		19.7
	cyper + PBO (50)	3.28 (2.55–4.20)	1.83 (0.24)	1.14	17.3
	cyper + DEF (50)	4.81 (3.84–6.07)	2.14 (0.32)	0.78	25.3
	cyper + TCPB (50)	1.74 (1.43–2.13)[e]	2.69 (0.35)	2.16	9.16
	cyper + 1-NPA(20)	1.84 (1.27–3.00)[e]	2.99 (0.48)	2.04	9.68
	cyper + 2-NPA (20)	2.08 (1.80–2.48)[e]	3.31 (0.54)	1.80	10.9
	cyper + *cis*-TPPA (30)	1.75 (1.32–2.69)[e]	3.54 (0.60)	2.14	9.21
	cyper + *trans*-TPPA (40)	1.42 (1.17–1.76)[e]	2.49 (0.38)	2.64	7.47
	cyper + *p*-NPPA (50)	1.11 (0.91–1.36)[e]	2.41 (0.35)	3.34	5.84
OP-R	prof alone	52.3 (46.0–59.2)	3.88 (0.57)		18.0
	prof + PBO (50)	67.7 (58.8–79.8)	2.89 (0.41)	0.77	23.3
	prof + DEF (50)	59.5 (42.5–91.2)	3.72 (0.56)	0.88	20.5
	prof + TCPB (50)	8.35 (6.29–10.3)[e]	2.84 (0.43)	6.26	2.88
	cyper alone	2.46 (1.86–3.43)	6.49 (0.88)		12.9
	cyper + 1-NPA (20)	0.84 (0.55–1.26)[e]	2.84 (0.41)	2.93	4.42
	cyper + 2-NPA (20)	0.68 (0.56–0.81)[e]	3.14 (0.43)	3.62	3.58
	cyper + *cis*-TPPA (30)	0.67 (0.56–0.78)[e]	3.12 (0.47)	3.67	3.53
	cyper + *trans*-TPPA (40)	0.70 (0.42–1.05)[e]	3.17 (0.42)	3.51	3.68
	cyper + *p*-NPPA (50)	0.79 (0.53–1.14)[e]	2.59 (0.39)	3.11	4.16

[a] Strains: LSU-S, insecticide susceptible; OP-R, profenofos selected; PYR-R, cypermethrin selected. [b] Treatments: cyper, cypermethrin; prof, profenofos. [c] FD = 95% fiducial limits. [d] SR defined as LD_{50} of insecticide alone/LD_{50} of insecticide plus synergist. [e] Value is significantly different from that of the insecticide alone treatment.

Table 4. Esterase Activities toward Pyrethroid and Nonpyrethroid Esters[a]

	strain[b]				
substrate	LSU-S	OP-R	R/S	PYR-R	R/S
1-NA	73.5 (9.51)^B	91.5 (12.9)^A	1.25	85.1 (15.9)^A	1.16
TPA	345 (79.5)^B	582 (126)^A	1.68	621 (158)^A	1.80
SMTB	98.5 (15.5)^C	214 (33.9)^A	2.17	188 (40.8)^B	1.91
1-NPA	16.6 (2.19)^C	39.6 (5.55)^B	2.39	50.9 (3.47)^A	3.07
2-NPA	12.6 (3.24)^B	35.2 (7.09)^A	2.12	38.6 (3.28)^A	3.06
cis-TPPA	0.82 (1.62)^C	2.02 (0.69)^B	2.46	6.88 (0.87)^A	8.39
trans-TPPA	1.43 (1.64)^C	6.57 (1.10)^B	4.59	10.6 (1.37)^A	7.41
p-NPPA	0.42 (0.36)^B	1.21 (0.65)^A	2.88	0.96 (0.72)^A	2.28

[a] Values represent mean activities (nmol min^{-1} mg $prot^{-1}$; ± SD) based on triplicate assays with 30 larvae from each strain. For comparing activities toward each substrate, values with the same letters are not significantly different (ANOVA–Tukey's HSD test, $P \leq 0.01$). [b] Strains: LSU-S, reference susceptible strain; OP-R, profenofos selected; PYR-R, cypermethrin selected.

Esterase Activities toward Pyrethroid and Nonpyrethroid Substrates. Regardless of the substrate used, activities were significantly greater (Tukey's HSD; $P \leq 0.01$) in larvae from the resistant rather than the susceptible strains (**Table 4**). In addition, esterase activities toward the nonpyrethroid substrates (1-NA, TPA, and SMTB) were significantly greater (Tukey's HSD; $P \leq 0.01$) than those measured with the pyrethroid esters (**Table 4**). For all three strains, activities were greatest in assays with TPA and lowest toward *p*-NPPA. However, differences in activities toward nonpyrethroid esters between the susceptible

and the resistant strains were not dramatic and ranged from 1.16- (for 1-NA) to 2.17-fold (for SMTB) in the PYR-R and OP-R strains, respectively. Also, activities toward nonpyrethroid substrates were generally similar between the insecticide resistant strains, although the activity with SMTB was statistically greater in the OP-R than the PYR-R strain. Differences in activities toward pyrethroid esters between resistant and susceptible strains were greater than those measured with nonpyrethroid esters and ranged from 3.1- and 2.8-fold (for 2-NPA) to 8.4- and 2.7-fold (for *cis*-TPPA) in the PYR-R and OP-R strains, respectively. In addition, in tests with the LSU-S strain, no activities were detectable toward *cis*-TPPA, *trans*-TPPA, and *p*-NPPA in five, four, and one of the tested individuals, respectively. Finally, esterase activities toward three of the pyrethroid substrates (1-NPA, *cis*-TPPA, and *trans*-TPPA) were significantly greater in PYR-R larvae than those from the OP-R strain, and differences ranged from 1.3-fold for 1-NPA to 3.4-fold for *cis*-TPPA.

DISCUSSION

Currently available bioassays provide little insight into the biochemical mechanism underlying resistance. Knowledge of the mechanism expressed in resistant pests would allow countermeasures to be tailored toward the mechanism being expressed in resistant members of pest populations. Synergist bioassays are commonly used to test for the involvement of metabolic resistance mechanisms; however, the specificity of synergists for detoxifying enzymes associated with resistance is often questionable (*3*, *29*, *30*). In addition, synergists have been shown to affect insecticide toxicity via mechanisms that do not involve metabolism (e.g., decreasing or increasing cuticular penetration; *30*−*32*). In the present study, we tested the hypothesis that compounds with structural similarity to pyrethroid insecticides will be more specific synergists of pyrethroid toxicity than compounds that are structurally unrelated.

Selection of larval *H. virescens* with either cypermethrin or profenofos resulted in cross-resistance to an array of insecticides, some of which act at differing target sites. Both OP-R and PYR-R larvae expressed resistance to profenofos, an organophosphorus insecticide that inhibits the enzyme, acetylcholinesterase, and also to pyrethroid insecticides, which alter function of voltage sensitive sodium channels. This aspect of the resistance profile suggests that a metabolic mechanism is at least partially associated with resistance in these strains. In tests with the OP-R strain, a high level of resistance (over 15-fold) was also measured to indoxacarb, which is a blocker of insect sodium channels (*33*) and alters the functioning of nicotinic acetylcholine receptors in mammals (*34*). This finding was unexpected because these insects had not been exposed to this compound. However, very high levels of resistance to indoxacarb have been reported in obliquebanded leafroller, *Choristoneura rosaceana*, prior to significant exposure to this insecticide (*35*, *36*).

Varying degrees of cross-resistance among the pyrethroids tested were measured in larvae from both resistant strains. In cypermethrin-selected, PYR-R larvae, resistance to pyrethroids with 3-phenoxybenzyl alcohols (i.e., cypermethrin and permethrin) was greater than to pyrethroids lacking this group (bifenthrin, *trans*-fenfluthrin, and tefluthrin). Similar results were observed in tests with OP-R larvae, suggesting that the phenoxybenzyl group of cypermethrin and permethrin contains targets for metabolism in these strains. This finding is consistent with those from previous studies showing that NADPH-dependent ring hydroxylation at the 2′- and 4′-positions of the phenoxybenzyl alcohol is a predominant route of pyrethroid metabolism by insecticide resistant *H. virescens* (*37*, *38*). Further support for involvement of P450 monooxygenases (P450 MOs) in resistance in the OP-R and PYR-R strains was measured in synergist assays, where coapplication of TCPB, a P450 MO inhibitor, significantly increased the toxicity of cypermethrin. In contrast, PBO, which has been widely used to ascertain the involvement of P450 MOs in resistance, had a lesser, nonsignificant effect on cypermethrin toxicity in the resistant strains. This result is similar to those from previous reports (*10*, *15*) and underscores the need to use multiple synergists in "diagnostic" bioassays (*3*).

Results from the current study also support previous findings that enhanced ester hydrolysis contributes to resistance in *H. virescens* (*3*, *39*−*41*). In a previous study, products of hydrolysis were the predominant metabolites following topical application of cypermethrin to pyrethroid resistant larvae from field-collected and laboratory-selected strains of this insect (*41*). In current tests, pyrethroid esters were among the most active synergists of cypermethrin toxicity in the PYR-R strain. Synergism was greatest with *p*-NPPA, which increased cypermethrin toxicity by 3.34-fold and reduced the RR in these insects from 20- to 5.8-fold. The residual resistance measured may reflect the action of additional mechanisms (e.g., P450 MO or reduced target site sensitivity). The expression of oxidase-based resistance is suggested based on the activity of TCPB as a synergist; however, the effect of this compound on esterases has not been examined directly. Alternatively, the doses of synergists used for these tests, which were chosen based on tests using larvae from the LSU-S strain, may have been suboptimal (i.e., too low) for larvae from the resistant strains. Thus, results measured may reflect incomplete inhibition of esterases.

A role for esterases in resistance in these strains is also supported by results from biochemical assays. Esterase activities toward a structurally diverse array of substrates were higher in both PYR-R and OP-R strains as compared with those in LSU-S larvae. Whereas activities in all strains were higher toward nonpyrethroid esters (i.e., 1- NA, TPA, and SMTB) than pyrethroid esters, differences in activities between susceptible and resistant strains were greater in assays with the pyrethroid esters. Furthermore, in comparisons between resistant strains, esterase activities toward 1-NPA and *cis*- and *trans*-TPPA were higher in PYR-R than OP-R larvae. These data suggest that substrate selectivities of esterases differ among resistant and susceptible larvae and that esterase activities associated with resistance to pyrethroid and organophosphorus insecticides may be measured with substrates that are structurally similar to the insecticide that is resisted. Because insecticide metabolism was not measured in the current study, our conclusions are necessarily tenuous and further studies are required. However, these results provide a foundation for subsequent efforts for use of such compounds for development of rapid assays to detect esterase-based resistance in field-collected *H. virescens*.

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